STAIN TECHNOLOGY

VOLUME 10 JANUARY, 1935 NUMBER 1

PROGRESS IN THE STANDARDIZATION OF STAINS

THE BACTERIOSTATIC USE OF DYES

Just five years ago an editorial in this journal discussed "Dyes for Bacteriostatic Work" (Stain Techn., 5, 1. 1930). At that time it was stated that the use of dyes in culture media for the sake of their bacteriostatic effect was a field of growing importance, but that the Stain Commission was not then testing or approving dyes for such purposes. It was stated that there were two reasons why dyes were not yet standardized on the basis of their bacteriostatic properties. The first of these reasons was the amount of work required and the lack of the necessary assistance on the part of the Stain Commission. The second was the fear that the certification of a stain for bacteriostatic purposes might be construed as implying its approval for medicinal use. This was because at the time of the editorial just cited the bacteriostatic properties of dyes were associated very largely in the minds of bacteriologists with their use as medicinal reagents.

In this last particular the situation has now considerably changed, because of the constantly increasing number of procedures for differentiating bacterial types which call for the use of dyes as ingredients of culture media. This, of course, is not a new idea, as several dyes, such as brilliant green, had been employed for this purpose for a good many years before it was suggested to employ dyes as antiseptics. The real change in the situation since 1930 is not, therefore, due to the introduction of any new idea but rather to the increase in the number of applications of the principle that had been proposed earlier. Hardly a month goes by at present without some bacteriologist suggesting a new medium in which some dye is included as one of its most important ingredients.

The question has naturally been raised again and again as to whether any degree of uniformity can be expected in the various batches of any one of these dyes that are put on the market. Altho the Stain Commission is doing its best to secure uniformity for such dyes when used as stains, there is as yet no guarantee that two different batches will have the same bacteriostatic properties. In fact, whenever any individual has carried on an investigation of this point he has obtained results indicating that such uniformity does not

exist. All this has pointed to the need of standardization of dyes for such purposes, carried out by methods similar to those used in their standardization as stains.

Fortunately, at the present time coöperative work is being planned with the American Public Health Association which may well overcome the first of the two difficulties mentioned in the previous editorial; namely, the lack of sufficient assistance to carry on the necessary investigations. The Laboratory Section of the American Public Health Association has recently appointed several committees on standard methods, one of which is on Diagnostic Procedures and Reagents, of which Dr. W. D. Stovall is chairman. These standard methods committees carry on their work, as is well known, by means of referees appointed in each particular field. Dr. Stovall has recently appointed Dr. Edmund K. Kline of Olean, N. Y. as referee on the subject of toxicity of dyes for bacteria. Arrangements have been made for close cooperation between Dr. Kline and the Commission on Standardization of Biological Stains.

The first result of this coöperation has been a report on brilliant green which was presented by Dr. Kline at the Pasadena meeting of the A. P. H. A. This report shows that differences in the toxicity of different batches of this dye actually exist. It also points out methods that can be satisfactorily employed in determining the bacteriostatic titre of a dye. Dr. Kline is expecting to pursue similar investigations on the subject of other dyes and it is very much hoped that as a result of this work standardization of dyes for their bacteriostatic properties can be brought about before many years.

—H. J. Conn.

The Certification of Stains (*continued*)

In the last number of this journal (Vol. 9, pp. 113–5) a brief discussion was given of some of the problems that have presented themselves in the standardization of certain biological stains. The stains thus discussed in the last number of the journal were brilliant cresyl blue, brilliant green, cresyl violet, and eosin alcohol soluble (ethyl eosin). In continuation of this discussion, there follow below brief notes on a few of the other stains which have been studied by the Stain Commission and eventually put on the certification basis.

EOSIN Y

The eosin group of stains have been about as badly mislabeled in the past as any that have been on the market. Eosin Y, frequently denoted yellowish eosin or eosin water soluble, has been sold under as great a variety of designations as any, but as a type it is quite distinct. Its characteristics were recognized quite early in the work on standardization and no serious difficulties presented themselves in connection with this dye.

The only problems concerning eosin that have ever arisen have come from the mislabeling above mentioned. It has sometimes happened that some investigator obtained good results using some prewar dye labeled eosin without being able to duplicate his findings with any eosin Y on the market after the war; but upon looking into the matter it has proved that the dye which he was accustomed to was actually something else, and good results have been obtained when the correct dye has been substituted for eosin Y. This confusion has occurred most notably in the case of phloxine.

EOSIN B

One of the problems that was called to the attention of the Stain Commission in the beginning of its work related to eosin B or, as it is sometimes called, bluish eosin. This dye is not very often called for in biological work but has its occasional applications. It was found that some of the American samples sold under this name did not give good results. Upon investigation it proved that the unsatisfactory samples were mixtures of eosin Y with some bluer homolog, probably phloxine or rose bengal. True eosin B, on the other hand, is a distinctly different chemical compound—a nitro derivative of eosin Y. As soon as this was called to the attention of stain manufacturers the correct compound was regularly supplied and there has apparently been no more difficulty in meeting the demands of biologists in this particular.

ERYTHROSIN

Comparatively few problems have been raised in the standardization of erythrosin. There are two dyes, one known as erythrosin Y, the other as erythrosin B, which are theoretically the di-iodo and tetra-iodo derivatives, respectively, of fluorescein. Mixtures of the two compounds, undoubtedly, are of more common occurrence than either alone and no evidence is yet at hand that one is more satisfactory to biologists than the other. The only problems that have occurred in the case of erythrosin are due to the mislabeling above mentioned. It is quite certain that in other countries, to this day, various types of eosin are on the market labeled erythrosin.

BASIC FUCHSIN

Basic fuchsin was one of the first three dyes to be studied by the Stain Commission; but in spite of all the study that has been given it, the dye cannot be said to be completely standardized for biological purposes even yet.

As has been frequently explained in reports from the Stain Commission, there are four primary basic fuchsins, differing from one another in the number of methyl groups they contain. Most basic fuchsins are mixtures of these four primary dyes, altho it is generally considered that pararosanilin represents the lowest and new fuchsin the highest homolog of the series. These four compounds are all quite similar, not only in chemical properties but also in shade, new fuchsin at one end of the series being only slightly bluer than pararosanilin at the other end.

Another well-known chemical difference between different samples of basic fuchsin is the form of the salt in which they occur. Chlorides and acetates are most commonly encountered, while some samples of basic fuchsin are mixtures of the chloride with the acetate.

Altho basic fuchsin is employed for a considerable number of different purposes in biological work, the Stain Commission quite early began giving its chief attention to two particular uses, namely, (1) for staining the tubercle organism, and (2) as a constituent of the Endo medium for differentiation in the colon-typhoid group of bacteria. There proved to be no great difficulty in securing a basic fuchsin satisfactory for the first of these two purposes. As a matter of fact, nearly all samples were satisfactory except for the fact that new fuchsin proved to be slightly too blue in shade to contrast properly with the counterstain methylene blue.

In the case of the Endo medium, the situation was different. Until recently it was very puzzling as to why some samples were satisfactory and others were not. Efforts were made to correlate these differ-

ences with the chemistry of the dye, both in the matter of the dye radical itself and that of the acid with which it is combined. These efforts were not successful, nor were those which endeavored to show the fault to be due to impurities. Finally, only about a year ago[1] it was shown that the irregularity linked up with the difficulty of getting some samples properly into solution in alcohol and that if a slight change in the standard Endo formula is adopted, practically any sample of basic fuchsin can be used for this purpose.

This would seem to standardize basic fuchsin, except that of recent years this dye has been called for in a new staining procedure which has presented problems of its own. This is the Feulgen reaction, a staining technic which really seems to be a microchemical test for aldehyde-like substances in tissue. Only a very few samples of basic fuchsin have been found to give a satisfactory Feulgen reaction. In fact, almost none of those at present on the market are satisfactory in this procedure. Accordingly, for this particular purpose basic fuchsin has not yet been standardized. Recent investigations, however, do indicate that the solution of this problem is more nearly at hand than ever before and it is hoped than an important announcement on the subject can be made in the near future.

ACID FUCHSIN

The difficulty in the standardization of acid fuchsin has arisen from its indefinite chemical nature. The chemistry of this dye is complicated by the fact that it may be manufactured from any mixture of the four primary basic fuchsins by means of sulfonation; and in this latter process either one, two or three sulfonic acid radicals may be introduced. As a result, there are twelve primary acid fuchsins theoretically possible, and they may be mixed in almost any combination.

The most critical test of acid fuchsin seems to be its behavior in the Van Gieson connective tissue stain, in which it is used in combination with picric acid, following staining in hematoxylin. Fortunately, nearly all acid fuchsin samples can be used in this technic. Some lots are unsatisfactory, however, and no significant correlation has been established as yet with either the chemical nature of the basic fuchsin from which the dye was prepared, or with the amount of sulfonation accomplished in its preparation. Accordingly, acid fuchsin cannot be said to have been standardized as yet on a truly scientific basis.

—H. J. Conn.

(To be continued)

[1]Conn, H. J. and Darrow, Mary A. Can the Endo medium be standardized? Stain Techn., 9, 61–9. 1934.

STAINS RECENTLY CERTIFIED

In the table below is given a list of the batches of stain approved since the last one listed in the October number of this journal.

STAINS CERTIFIED SEPTEMBER 1, 1934 TO NOVEMBER 30, 1934*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Congo red	NQ 5	94%	As histological counterstain	Sept. 4, 1934
Methyl green	CG 6	73%	As histological stain and as constituent of Pappenheim stain	Sept. 5, 1934
Sudan IV	NZ 6	88%	As fat stain	Sept. 12, 1934
Giemsa stain	NGe 1	——	As blood stain	Sept. 20, 1934
Martius yellow	NYm 3	78%	As histological counterstain	Oct. 3, 1934
Malachite green	NMg 4	76%	As histological and bacteriological stain	Oct. 3, 1934
Rose bengal	NRb 2	90%	As bacteriological stain	Oct. 4, 1934
Wright stain	LWr 5	——	As blood stain	Oct. 17, 1934
Crystal violet	LC 8	94%	As histological, cytological and bacteriological stain	Oct. 17, 1934
Methylene blue	LA 5	85%	For histology and bacteriology, and as constituent of blood stains	Oct. 22, 1934
Wright stain	NWr 9	——	As blood stain	Oct. 29, 1934
Basic fuchsin	NF 26	92%	For histology and bacteriology, and for use in Endo medium	Nov. 1, 1934
Nile blue sulfate	NNb 2	90%	As stain for fats and fatty acids	Nov. 19, 1934
Anilin blue WS	NK 3	——	As histological stain	Nov. 28, 1934

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its common uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

REACTIONS OF HEMOGLOBINIFEROUS CELLS TO ACID AND BASIC DYES UNDER VARYING CONDITIONS OF H-ION ACTIVITY

JAMES E. KINDRED, *Laboratory of Histology and Embryology, Medical School, University of Virginia, Charlottesville, Va.*

ABSTRACT.—**1. An attempt has been made to apply Loeb's concept of the amphoteric nature of proteins for the discrimination of suspected hemoglobiniferous substances from known hemoglobiniferous substances according to their reactions to acid and basic dyes with reference to the isoelectric point of hemoglobin (pH 6.8).**

2. The substances in the cytoplasm of known hemoglobiniferous cells (red blood corpuscles, normoblasts and erythroblasts) of the lymph nodes, spleen and bone marrow of the albino rat, when suspended in buffered dye-sucrose solutions, retain eosin on the acid side of pH 7.0, but the substances of the Russell bodies, suspected of being hemoglobiniferous, do not retain eosin at all; and the cytoplasm of the plasma cells, also alleged to be slightly hemoglobiniferous, only retains eosin on the acid side of pH 6.4.

3. The only basic dye used which did not precipitate in buffer solutions was methylene blue. This did not react with hemoglobin in accordance with Loeb's concept, because it did not penetrate mature red blood corpuscles and in those immature erythrocytes which it did penetrate it was precipitated by the reticulum.

4. Therefore, from the results obtained with the acid dye, it is tentatively concluded that the substance in the Russell bodies and in the cytoplasm of the plasma cells are not hemoglobiniferous because they do not react as do the substances in known hemoglobiniferous cells with reference to the isoelectric point of hemoglobin.

5. More investigation, however, must be carried out on both fresh and fixed material before a final unequivocal answer can be made to this problem.

It is a well-known fact that Russell bodies occur within the cytoplasm of plasma cells. These bodies in fixed tissues have an acidophilic reaction with acid dyes and a selective reaction with acid dyes in such staining mixtures as eosin-azure-II. According to Debreuil and Favre (1921), who reviewed the interpretations as to the chemical nature of these bodies, no satisfactory explanation has been advanced which withstands critical analysis. From their own investigations they suggest that the Russell bodies are stored secretion products of plasma cells. Jordan and Speidel (1929) and Jordan (1929)

suggest that the Russell bodies are hemoglobiniferous because their tinctorial reactions resemble those of hemoglobin and they are developed within the cytoplasm of plasma cells which are regarded as abortive erythroblasts. The author (1932) also concluded that they are hemoglobiniferous, because of the morphologic similarity of many of them to hemoglobin crystals; the similarity of their tinctorial reactions in Helly-fixed tissues to those of the cytoplasm of the red blood corpuscles, free hemoglobin and heme in aqueous solutions of acid dyes buffered to different levels of H-ion activity; and the similarity of their reactions to these same materials in tissues fixed in Helly's fluid and subjected to Okajima's phosphomolybdic acid-alizarin technic which Okajima (1916) claims as specific for hemoglobin. Since these conclusions were made from observations gained by the study of fixed tissues and there remained the possibility that the fixation may have produced changes in the Russell bodies which caused them to react as above, the present investigation of their reactions in fresh tissues was undertaken.

The working hypothesis of this investigation is based upon the concept of hemoglobin as an amphoteric protein which predominates in hemoglobiniferous cells and as such has a definite isoelectric point (pH 6.8 for reduced hemoglobin, Stadie and Martin, 1924) on the acid side of which it should act like a cation and unite with the color radical or anion of acid dyes, and on the alkaline side of which it should act like an anion and unite with the color radical or cation of basic dyes. The hypothesis was suggested from the conclusions of Loeb (1924) drawn from experiments with gelatin and other pure proteins, and particularly those experiments in which the proteins on the acid side of their respective isoelectric points retained acid dyes and on the alkaline side, basic dyes. Since in the present investigation, the experimental material is heterogeneous as regards cell types and the proteins lie within cell membranes, it is necessary to assume that by changing the H-ion concentration of the environmental medium there is also a change in the pH of the cell interior in the same direction, and that consequently the ionogenic activities of the cell proteins are modified. These assumptions are made from the evidence given by Van Slyke et al. (1923) that changes in the pH of the medium in which red blood corpuscles are suspended produces similar changes in the pH of the interior of the corpuscles, altho the interior of the corpuscle is always slightly less alkaline than the surrounding medium. Accordingly, in applying these views for the discrimination of hemoglobin in living cells which are known or suspected of being hemoglobiniferous, positive reactions to acid dyes on

the acid side of the isoelectric point of hemoglobin (pH 6.8), and negative reactions to the same dyes on the alkaline side of this point, would indicate the presence of hemoglobin. Comparable reactions with basic dyes, but in the reverse direction, would further confirm such a conclusion.

Jodlbauer and Haffner (1921) have shown that rose bengal and eosin are retained better by red blood corpuscles in acid solutions than in alkaline solutions, but they did not relate this condition of retention to any specific protein in the corpuscles. Other investigators (Wiechmann, 1921; Höber and Memmesheimer, 1923; Seo, 1923; Tanaka, 1924; and Jurišić, 1927) have been concerned with the permeability of the membrane of the red blood corpuscle to acid and basic dyes under different physico-chemical conditions with particular reference to the lipoid solubility of the dyes. Bornstein and Rüter (1925) have sought to show that basic dyes penetrate red blood corpuscles and enter into combination with the cell proteins and that this combination can be broken down by alkaloid salts. But none of these investigators has attempted to use the dyes for the discrimination of specific substances such as hemoglobin. Furthermore, most of these investigators used indirect colorimetric methods to measure the degrees of penetration of the dyes, methods which cannot be used for the discrimination of intracellular substances, because of the heterogeneous nature of the cells composing the lymph nodes, spleen and bone marrow. In the method used here, the presence of the dye is determined by direct observation of the cells and the degrees of retention of the dye are compared for the different cells with parti ʻır reference to the isoelectric point of hemoglobin.

MATERIAL AND METHODS

The materials used were submaxillary lymph nodes, spleen and bone marrow of adult albino rats (Wistar stock). The rats were usually killed by a blow on the head, but some were anesthetized. The tissue to be used was removed as rapidly as possible from the body and teased with glass needles in glass dishes (in water bath, 37° C.) containing 5 cc. of 7.0% sucrose buffered to different degrees of H-ion activity, the pH of which was determined with a Youden electrical H-ion apparatus. The volume of each of the tissues was quite small in comparison with the volume of the medium and had no effect on the pH of the solution in which it was suspended. After teasing in sucrose solution, the suspension was centrifuged for two minutes, the sucrose was poured off and replaced with dye-sucrose solution of the same concentration and pH. The temperature was

maintained as nearly constant as was possible (37° C.). A sample of the mixture was tested for the pH and this reading was recorded as the pH of the particular experiment. The sucrose solutions were hypertonic. The buffer solutions were made from Kolthoff's buffer tablets (pH 3.0–5.4, borax and succinic acid; 5.6–7.4, phosphates; 7.4–9.0, borax-sodium bicarbonate) and 5 cc. of the buffer solutions were added to 15 cc. of the dye-sucrose solution to bring the volume up to 20 cc. The dyes used were: eosin Y (Coleman and Bell, CE-5; 84% dye content); light green (Grübler); fuchsin (Grübler); Bordeaux red (Grübler); methylene blue (Nat. Anil. and Chem. U. S. P. Med., NA-6; 82% dye content). The concentrations at which the dyes were used are given in the text.

The suspension of the tissue in the dye-sucrose was allowed to stand with stirring for 15 minutes in an incubator at 37° C. It was then centrifuged for two minutes for concentration of the tissue, and the supernatant solution was poured off. A sample of the concentrated tissue was withdrawn with a capillary pipette and transferred to a slide in a warm stage (37° C.) and covered with a cover slip rimmed with vaseline. To the tissue remaining in the centrifuge tube was added 5 cc. of sucrose of the same concentration, temperature and pH. This was centrifuged and after the supernatant solution was drawn off, a sample of the tissue was mounted as above. Controls were run without the dye. The samples mounted in the warm stage were studied for 15 to 30 minutes with the oil immersion lens. All records were made from the washed preparations.

The degree of retention of the dye by the cells was estimated by comparing known solutions of the dye contained in a hemocytometer chamber (100 μ deep) with the content of dye in the cytoplasm of the cell. The comparator slide was held above the ocular of the microscope and the cell was viewed thru it. At first it was thought that a definite quantitative estimate could be made by this method and the results were recorded in terms of dye percentage as corrected for the difference between the depth of the comparator and the diameter of the cell or cell granule, but it was found that the eye could not discriminate with sufficient precision to make the color differences of value, consequently the results have been transposed into the positive signs as used in the tables.

OBSERVATIONS

Before giving a description of the results it seems necessary to make a statement concerning the method used for discriminating between the cell types listed in the tables. There is no confusion in the identi-

fication of red blood corpuscles, Russell body cells and lymphocytes in fresh preparations. The red blood corpuscles are non-nucleated, yellowish colored, biconcave discs, which in hypertonic solutions show wrinkling or crenation of the cell membrane. The Russell body cells have a content of large or small colorless spheres, polyhedra, rods or ovoid bodies. The nucleus of these cells is usually hidden, but when seen is small and clear. These cells vary in size from 8 to 20 μ in diameter, depending upon the number and size of the contained bodies. The colorless character of the Russell bodies is in sharp contrast with the contents of the macrophages, the irregularly shaped debris of which is usually deeply yellow and shiny. This material is interpreted as hemosiderin. The nucleus of the macrophage is large and clear. The lymphocytes of small, medium and large size are characterized by a clear or slightly granular, grayish cytoplasm encircling as a narrow rim a large clear nucleus in which may be present a visible nucleolus. In hypertonic or acid solutions the block-like arrangement of the chromatin becomes visible.

The normoblasts are characterized by their small size (6 to 7 μ in diameter), dense shiny nucleus, and rim of clear yellowish cytoplasm which tends to overshadow the nucleus. Cells like the normoblasts, but larger, and with a larger nucleus and less cytoplasm are interpreted as erythroblasts. In hypertonic or acid solutions the chromatin appears to be distributed thruout the nucleus in the form of fine granules. Normoblasts and erythroblasts occurred in the spleen and bone marrow, but were never seen in the lymph nodes. In contrast with the erythroblasts, the plasma cells have a smaller nucleus and a relatively greater amount of cytoplasm which has a grayish tinge and is either dense or contains very fine granules closely packed together; sometimes vacuoles are present. A clear juxtanuclear area usually occurs in the plasma cells; the nucleus is eccentrically placed and the cell boundary is sharp and clear. These cells may be subtriangular, or almost quadrilateral in shape, suggesting a very dense cell content. In hypertonic or acid solutions the nuclear chromatin can be seen to be arranged in typical "radkern" fashion with a ring of chromatin blocks attached to the nuclear membrane and a central block or blocks.

The hemocytoblasts cannot be distinguished from the large lymphocytes in fresh preparations. The myelocytes have a more or less bean-shaped nucleus with a content of fine but distinct granules. In the premyelocytes the granules are coarser and the nucleus is partially lobulated. In the neutrophils the nucleus is multi-lobulated and the cytoplasm finely granular. The nucleus of the eosinophilic granulocyte is large and bilobed, and the cytoplasm is filled with coarse

spherical or rod-shaped granules which have a yellowish tinge. The granules of the mast cells are always spherical and are very regularly arranged thruout the cytoplasm of the cell; the nucleus is difficult to see and is smaller than that of any of the other granulocytes.

I. Acid Dyes. a. eosin y.

1. *Lymph nodes.* In Table 1 are shown the results of exposure of the cells of teased submaxillary lymph nodes to eosin Y (0.125%) in hypertonic sucrose at different levels of H-ion activity. It will be noted that the cytoplasm of the red blood corpuscles showed a faint trace of the dye on the alkaline side of the isoelectric point of hemoglobin.

Table 1—Reactions of Cytoplasm of Cells of Submaxillary Lymph Nodes to Eosin Y (0.125%) in Sucrose (7.0%) Buffered to Different Levels of H-ion Activity. Time 15 Minutes. Temp. ca. 37° C. Degree of Reaction Indicated by Positive Sign. Preparations from 22 Animals

pH	Red blood corpuscles	Russell bodies	Plasma cells	Lymphocytes
6.2	+ + +	0	+	0
6.4	+ +	0	+	0
6.6	+ +	0	0	0
6.8	+	0	0	0
7.0	+	0	0	0
7.2	0	0	0	0

The pH of the solution in which this occurs is 7.0, but the pH of the cell interior is actually lower than this by at least 0.1 pH as has been pointed out in the introduction, so that actually in this reaction and in all of the others there is a slight difference between the pH recorded for the solutions and the pH of the interior of the cell. Accordingly, the reaction of the cytoplasm of the red blood corpuscle at pH 7.0 may be taken to indicate the retention of a small amount of the dye by the hemoglobin in the region of its isoelectric point. When the acidity was increased, however, the retention of eosin became greater and when the alkalinity was increased, eosin was no longer retained. No other cells in the lymph nodes reacted in this manner. The Russell bodies did not react with eosin at all, neither did the lymphocytes. The plasma cells began to retain eosin on the acid side of pH 6.4.

2. *Spleen.* The reactions of the same groups of cells to eosin were practically identical with those of the lymph nodes (Table 2). In ad-

dition, observations were made of the reactions of the normoblasts, erythroblasts and macrophages. The cytoplasm of the normoblasts and erythroblasts showed the same degrees of eosin retention as the cytoplasm of the red blood corpuscles in the same range of pH. The Russell bodies remained colorless and the cytoplasm of the plasma cells started to retain eosin on the acid side of pH 6.4. The debris of the macrophages for the most part remained bright yellow, but some of the newly ingested red blood corpuscles showed reactions to eosin comparable to the reactions of the free red corpuscles.

TABLE 2—REACTIONS OF CYTOPLASM OF CELLS OF THE SPLEEN TO EOSIN Y (0.125%) IN SUCROSE (7.0%) BUFFERED TO DIFFERENT LEVELS OF H-ION ACTIVITY. TIME 15 MINUTES. TEMP. CA. 37° C. DEGREE OF REACTION INDICATED BY POSITIVE SIGN. PREPARATIONS FROM 27 ANIMALS.

pH	Red blood corpuscles	Normoblasts	Erythroblasts	Russell bodies	Plasma cells
6.2	+ + +	+ + +	+ + +	0	+
6.4	+ +	+ +	+ +	0	+
6.6	+ +	+ +	+ +	0	0
6.8	+	+	+	0	0
7.0	+	+	+	0	0
7.2	0	0	0	0	0

3. *Bone marrow.* In Table 3 are shown the results of the action of eosin on the cells of the bone marrow. As in the spleen, the cells of the hemoglobiniferous series showed comparable reactions to eosin. The plasma cells and Russell bodies reacted as in the lymph nodes and spleen. The debris of the macrophages also reacted as in those organs. No distinction could be made between lymphocytes and hemocytoblasts, since the cytoplasm of both of these types of cells did not react with eosin. The myelocytes with few granules showed a faint reaction with eosin up to pH 6.8. The eosinophils, however, contained granules which reacted very strongly with eosin up to pH 7.4. These granules were the first cytoplasmic substances to stain and they retained the eosin in more alkaline pH than any other cytoplasmic constituent. Some of the granules were rod-shaped and others were spherical. The granules of the mast cells did not react with eosin.

In order to see what effect the concentration of the dye might have upon the tinctorial reactions of the cytoplasm several series of experiments were made on the cells of the bone marrow. These cells were put into sucrose solutions at pH 7.0 and pH 6.6, respectively, and the co-

centration of the dye varied from 0.005% to 0.05%. No reactions were observed in any of the cells after 30 minutes until a concentration of 0.02% was reached in the pH 7.0 series. At this concentration a faint trace of eosin was present in the eosinophilic granules. The red blood corpuscles, normoblasts and erythroblasts showed no reaction to the dye in the series of concentrations up to 0.05% at pH 7.0, but began to show a faint trace of the dye when the concentration was increased to 0.1% or approximately the concentration used in the other experiments. At pH 6.6, however, the hemoglobiniferous cells began to retain eosin when in a concentration of 0.01%, but it was never as strongly concentrated in these cells as in the eosinophilic granules.

TABLE 3—REACTIONS OF CYTOPLASM AND CYTOPLASMIC GRANULES OF CELLS OF THE BONE MARROW TO EOSIN Y (0.125%) IN SUCROSE (7.0%) BUFFERED TO DIFFERENT LEVELS OF H-ION CONCENTRATION. TIME 15 MINUTES. TEMP. CA. 37° C. DEGREE OF REACTION INDICATED BY POSITIVE SIGN. 26 ANIMALS

pH	Red blood corpuscles	Normoblasts	Erythro-blasts	Russell bodies	Plasma cells	Eosinophils
6.2	+ + +	+ + +	+ + +	0	+	+ + +
6.4	+ +	+ +	+ +	0	+	+ + +
6.6	+ +	+ +	+ +	0	0	+ + +
6.8	+	+	+	0	0	+ + +
7.0	+	+	+	0	0	+ +
7.2	0	0	0	0	0	+ +

These experiments show that the concentration of the eosin has something to do with its retention and that there is a degree of concentration at which it may penetrate, but cannot be seen by the eye; and the more acid the solutions the less the dye concentration needs to be in order that it may be retained in visible quantities by the cell. Furthermore, the substance in the eosinophilic granules seems to be more alkaline than hemoglobin in the range of pH studied, or in other words, the protein which composes them has a higher isoelectric point, probably in the vicinity of pH 7.4.

B. LIGHT GREEN, ACID FUCHSIN AND BORDEAUX RED

These dyes were used in molecular concentrations approximately equal to eosin as used above. The tissues were suspended in dye-sucrose solutions buffered to different pH which conformed in all respects to the conditions under which the eosin was used. No reaction, how-

ever, was observed between these dyes and any of the cells of the lymph nodes, spleen and bone marrow. No deplasmolysis of the red blood corpuscles was observed as would be expected to occur if these dyes had penetrated in the leuco condition. All of these dyes are practically lipoid insoluble.

II. Basic Dyes. a. methylene blue

In using methylene blue, no attempt was made to control the concentration in this first group of experiments to such a degree that the nuclei would be unaffected, because it was early realized that in order to get visible results the methylene blue had to be used in lethal dosage.

Table 4—Reactions of Cytoplasm and Cytoplasmic Granules of Cells of Submaxillary Lymph Nodes to Methylene Blue (0.03%) in Sucrose (7.0%) Buffered to Different Levels of H-ion Activity. Time 15 Minutes. Temp. ca. 37° C. Degree of Reaction Indicated by Positive Sign. 19 Animals

pH	Red blood corpuscles	Russell bodies	Plasma cells	Lymphocytes	Mast cell granules	Macrophage (debris)
6.8	0	0	+	+ +	+ + +	green
7.0	0	0	+	+ +	+ + +	"
7.2	0	0	+	+ +	+ + +	"
7.4	0	0	+ +	+ + +	+ + +	"
7.6	0	0	+ +	+ + +	+ + +	"
7.8	0	0	+ +	+ + +	+ + +	"
8.5	0	0	+ +	+ + +	+ + +	"

1. *Lymph nodes.* In Table 4 are shown the results of the reactions between methylene blue and cytoplasm of cells from the submaxillary lymph nodes. The dye apparently did not penetrate the mature red blood corpuscles even on the alkaline side of the isoelectric point of hemoglobin. The cytoplasm of the plasma cells began to react with the dye at pH 6.2, altho this is not shown in the table. With increasing pH the color of the plasma cells became progressively deeper as recorded in the table. The Russell bodies did not react with the methylene blue at any pH, altho the dye penetrated the cells and was retained by the intervening cytoplasm. The debris of the macrophages became green. The dye penetrated the lymphocytes readily and was retained by them thruout the whole range of pH studied in a much stronger concentration than in the plasma cells.

2. *Bone marrow.* In Table 5 the reactions between methylene blue and the cytoplasm of the cells of the bone marrow are shown. It will be noted that no discrimination as regards retention of the dye by lymphocytes as contrasted with hemocytoblasts can be made. Both types of cells tend to retain less dye as the surrounding medium is made more acid. The plasma cells retained the dye until pH 6.2 and on the acid side of this point they no longer reacted with it. In the stained plasma cells an unstained juxtanuclear area could be seen. As in the lymph nodes, the red blood corpuscles were apparently not per-

TABLE 5—REACTIONS OF CYTOPLASM AND CTYOPLASMIC GRANULES OF CELLS OF THE BONE MARROW TO METHYLENE BLUE (0.03%) IN SUCROSE (7.0%) BUFFERED TO DIFFERENT LEVELS OF H-ION ACTIVITY. TIME 15 MINUTES. TEMP. CA. 37° C. DEGREE OF REACTION INDICATED BY POSITIVE SIGN. 48 ANIMALS.

pH	Rbc	Norm.*	Eryth.*	Russ. bd.	Pl. c.	Lym.	Hemo-c'blt	My-elo-cyte	Mast. c. gran.	Macro. (debris)
4.0	0	+	+	0	0	+	+	0	++	green
4.8	0	+	+	0	0	+	+	0	++	"
5.8	0	+	+	0	0	++	++	+	+++	"
6.2	0	+	+	0	+	++	++	+	+++	"
6.4	0	+	+	0	+	++	++	+	+++	"
6.8	0	++	++	0	+	++	++	+	+++	"
7.0	0	++	++	0	++	+++	+++	+	+++	"
7.2	0	++	++	0	++	+++	+++	+	+++	"
7.4	0	++	++	0	++	+++	+++	+	+++	"
7.6	0	++	++	0	++	+++	+++	+	+++	"
7.8	0	++	++	0	++	+++	+++	+	+++	"
8.5	0	++	++	0	++	+++	+++	+	+++	"

*Reticulum only.

meable to the dye, but thruout the range of pH given in the table (pH 4.0 to 8.5) the dye penetrated immature non-nucleated red blood corpuscles (reticulocytes), normoblasts and erythroblasts and was precipitated on the reticulum. There was no diffuse staining of the cytoplasm such as occurred in lymphocytes and plasma cells, but the golden-yellow hemoglobin appeared to be unaffected and showed clearly as a background for the reticulum. The mast cell granules were the most reactive to the dye of any cytoplasmic constituent and retained it in high concentration thruout the whole series of prepa-

rations. The Russell bodies did not react with the dye. The macrophages contained greenish-blue fragments. The eosinophilic granules were not affected by the dye, while the fine granules of the myelocytes, premyelocytes and neutrophils retained it faintly in the more alkaline solutions.

3. *Spleen.* The reactions of the cells of the spleen were the same as those of the bone marrow.

4. *Dilute solutions.* In addition to the above observations made from preparations in which methylene blue was used in lethal dosage as indicated by the staining of the nucleus, another series of experiments was done to find out what effect the dye concentration might have upon the retention of the dye by the cells at different levels of pH. Only a few experiments were made in this series. It was found that in a 0.005% solution of methylene blue in sucrose (7.0%) at pH 7.6, the nuclei and cytoplasm of the lymphocytes of the bone marrow stained deeply almost at once; the nuclei and cytoplasm of the plasma cells stained faintly; the nuclei of the normoblasts stained faintly, but no reticulum showed in the cytoplasm; and the mast cell granules stained deeply. In the same dye concentration at pH 7.0 only the mast cell granules reacted. Thus, as in similar experiments with eosin, it is evident that the reactivity of the cytoplasm of the cell with the dye is largely conditioned by the pH of the surrounding medium rather than by the concentration of the dye.

B. OTHER BASIC DYES

Gentian violet, neutral red, Janus green and brilliant cresyl blue were used in preliminary experiments, but were found to be unsatisfactory because of the manner in which they precipitated with changes in pH by the buffers.

III. Deplasmolysis Experiments

Other investigators have used the method of deplasmolysis to determine the penetration into cells of substances which cannot be seen by the eye. In this method the cells to be studied are first exposed to hypertonic solutions, and after they had shrunk because of changes in the water balance they were exposed to the same hypertonic solutions to which the substance whose penetration was to be studied was added (Jacobs, 1924). In the case of eosin, which does not undergo reduction changes, the penetration and retention is indicated by changes in the color of the cells. With methylene blue, however, there is the possibility that it may penetrate and be retained in the reduced leucobase phase. Therefore, since no visible penetration of methylene blue could

be seen in the red blood corpuscles in the above experiments, a series of deplasmolysis experiments was made to see whether there was evidence that the dye penetrated and was retained by the cytoplasm in the leucobase condition.

Small amounts of tissue from the bone marrow were placed in hypertonic solutions of sucrose buffered to different levels of H-ion activity and samples were withdrawn from time to time to see if the cells had changed from a wrinkled condition to a swollen deplasmolyzing condition. No changes were observed in the red blood corpuscles in these solutions over a period of four hours. These observations were compared with the appearances of red blood corpuscles suspended in hypertonic sucrose-methylene blue (0.043%) solutions. No changes were observed in the shrunken red corpuscles over a period of eight hours during which time each suspension was kept at 37° C. From these experiments it is concluded that methylene blue does not penetrate mature red blood corpuscles in either the colored or leuco phase.

DISCUSSION AND CONCLUSIONS

The essential facts which have been found by these experiments show that substances in the cytoplasm of known hemoglobiniferous cells (red blood corpuscles, normoblasts and erythroblasts) retain the acid dye, eosin, when suspended in hypertonic sucrose solutions of this dye at levels of H-ion activity on the acid side of the isoelectric point of hemoglobin (pH 6.8). The suspected hemoglobiniferous cells do not do this; the Russell bodies do not react with eosin at all and the cytoplasm of the plasma cells reacts with eosin only when the surrounding medium is more acid than pH 6.4. In these respects there has been demonstrated a reaction between eosin and hemoglobin which is suggestive of a chemical reaction such as Loeb (1924) found to exist between gelatin and acid dyes on the acid side of its isoelectric point. Attempts to confirm this reaction with several other acid dyes were unsuccessful because of the failure of these dyes (light green, acid fuchsin, and Bordeaux red) to penetrate the cells.

To confirm further the amphoteric nature of the substance in the hemoglobiniferous cells the experiments with methylene blue were undertaken, but the results show that several complicating factors prevent a demonstration of the union of methylene blue and hemoglobin on the alkaline side of the isoelectric point of the latter. In the first place, methylene blue does not apparently penetrate mature red blood corpuscles; and in the second place, when it penetrates the immature red blood corpuscles (reticulocytes), normoblasts and erythroblasts, there is such a strong retention of it by the reticulum that

no effect can be seen on the diffusely distributed hemoglobin. Apparently, the protein of the reticulum is so much more acid than the diffuse hemoglobin that all of the dye which enters the cell combines with it. The Russell bodies do not react with methylene blue, but the cytoplasm of the plasma cells does react, altho not so strongly as does the cytoplasm of the lymphocytes and mast cell granules.

The question arises whether the reactions obtained by this method are more specific for the determination of intracellular hemoglobin than are those used on fixed tissues. In fixed tissues the cytoplasm of any cell reacts according to the predominant acidity or alkalinity of its component substances as they are precipitated in fixation. Usually material fixed in Helly's fluid, a congerie of oxidizing, reducing and precipitating agents, the cytoplasm of the hemoglobiniferous cells reacts with certain acid dyes, but the reaction can only be said to be specific for hemoglobin in case certain cells lose or retain the dye at the same pH, when the pH of the dye solution or washing solution is varied. It was from such a series of experiments that I previously concluded that Russell bodies were hemoglobiniferous, because their tinctorial reactions varied in the same direction and to the same degree as did the cytoplasm of hemoglobiniferous cells (Kindred, 1932). But in the present series of experiments where the substances in the cells are apparently unmodified by fixation, the reactions of the Russell bodies are different from those of the cytoplasm of the hemoglobiniferous cells. Therefore, it may be concluded tentatively that fixation with Helly's fluid imparts certain chemical characteristics to the Russell bodies which make them react like fixed hemoglobin. The same thing could be said of plasma cells and erythroblasts which in fixed tissues react very much alike, but whose reactions in fresh tissues are quite different. Hence, it would seem doubtful that the Russell bodies are hemoglobiniferous. They may be inert products of degeneration which are stored in the plasma cells and which in Helly-fixed material react with the same tinctorial reactions as the substances in hemoglobiniferous cells. This view, however, cannot yet be considered as final, and more tests with different reagents and a variety of treatments of the tissues must be made before an unqualified answer can be given.

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A NOTE ON "GLYCHROGEL" MOUNTING SOLUTION

R. M. WOTTON and R. L. ZWEMER, *Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York*

The preparation of "glychrogel" mounting solution has been briefly described elsewhere,[1] together with a description of its usefulness in mounting frozen or gelatin-embedded sections. It is being used as a routine mounting solution by surgical pathologists; has been used for the preservation of 'teased' preparations in class room work; and has been used in our own research for over a year. One laboratory reports its value in mounting Marchi-stained sections which do not keep well in Damar. They run the slides down to water and then mount in glychrogel.

Because some workers have been unable to prepare a satisfactory solution we felt that added information as to its properties and constituents might be desirable.

For approximately 100 cc. of glychrogel it is necessary to use 80 cc. of distilled water, 20 cc. of pure glycerin, 3 gm. of Knox granulated gelatin, and 0.2 g. of chrome alum. The chrome alum must be potassium chromium sulphate ($K_2Cr_2(SO_4)_4 \cdot 24H_2O$).

Dissolve the chrome alum in 30 cc. of the water by aid of heat and then the gelatin in 50 cc. of hot water. Add the 20 cc. of glycerin to the gelatin solution with constant stirring and then add the warm, greenish chrome alum solution. The combined liquid should be heated as little as possible, as glychrogel is probably a colloidal solution which is denatured into an irreversible phase by excessive heating.

When thoroly mixed, filter rapidly and add a crystal of camphor as a preservative. The stock bottle should be well stoppered to prevent evaporation. If the solution congeals in a cold room, or if bubbles are present, the bottle should be left in an oven at 37° C. until the solution is clear and flows freely.

In putting on cover glasses it is best to leave a small quantity of the mounting medium around the edges of the coverslip. This slight excess forms a hard protective film very quickly and holds the preparation in place for immediate use. Too much, however, should not be used as this tends to form a thick preparation and renders it more susceptible to damage. Altho glychrogel dries rapidly around the edges where it is exposed to the air, it hardens but slowly in the center

[1]Zwemer, R. L. A method for studying adrenal and other lipoids by a modified gelatin embedding and mounting technique. Anat. Rec., **57**, 41–44. 1933.

of the preparation. On solidification it shrinks somewhat and thus draws the coverglass closer to the slide, a desirable feature in most cases, but an added reason for the use of a slight excess. If used with caution, thick cell-mounts of about 1 mm. in depth can be made.

The index of refraction of glychrogel increases as it solidifies, but the increase is not so great if the mounting solution has been heated too often. Duplicate measurements, on two batches designated A and B, were made by the method outlined by Chamot and Mason.[2] The indices of refraction were again determined after some days (Table 1). The optics employed in this method were a Zeiss 20× achromatic

TABLE 1—INDEX OF REFRACTION

Glychrogel A (excessive heating)		*Glychrogel B* (minimum heating)	
While liquid	1.36	While liquid	1.38
After hardening (5 days)	1.41	After hardening (7 days)	1.75
Increment in γ	.05	Increment in γ	.37

TABLE 2—REFRACTIVE INDICES OF MOUNTING MEDIA*

Air	1.00	Damar	1.52
Distilled water	1.33	Canada balsam	1.54
Glychrogel A	1.41	Methylene diiodide	1.74
Glycerin	1.46	Glychrogel B	1.75
Glycerin jelly	1.47	Hyrax	1.81

*In general substances of low refractive index are better revealed by mounting media of high refractive index and *vice versa*. Mann[3] says, "Some substances of high refractive index are converted into, apparently, lowly refracting ones by being mounted in substances possessing exceedingly high indices."

objective, 10× eyepieces and an achromatic condenser. It is to be remembered that the mathematical function of the fine adjustment of a microscope, if plotted, will be a sigmoid curve. The actual value therefore, of the divisions on the graduated head are not equal over the entire range of motion. To eliminate this factor we always started from one point and thus worked over the same segment of the curve in measuring D and d. The differential $\Delta s \longrightarrow \infty$ and so may be neglected.

With colloidal solutions it is difficult to exactly duplicate the properties of one batch with another. We feel, however, that with ordinary care a satisfactory aqueous mounting medium with a good index of refraction can be prepared for general use.

[2]Chamot and Mason. Handbook of Chemical Microscopy. Vol. I., p. 380. 1930.

[3]Mann, G. Physiological Histology. Oxford, Clarendon Press. p. 377. 1902.

TRICHLORETHYLENE AS A SOLVENT IN HISTOLOGICAL TECHNIC

R. E. Oltman, *Department of Botany, University of Minnesota, Minneapolis, Minnesota*

The increasing use of non-inflammable chlorinated hydrocarbons in industry to replace such solvents as benzene and toluene suggests their applicability in histological technic to replace xylene. The nausea and dizziness caused by xylene are well known to many histologists, as are the disadvantages of balsam-xylene solutions; namely, their slow drying properties and their gumminess in more concentrated solutions.

Trichlorethylene, $CHCl{:}CCl_2$, has been found to replace xylene advantageously in any histological operation. When used as a general clearing agent, no extraction of stains is observed. Buchner[1] has reported the complete solubility of paraffin in this solvent, and the author has found this solubility to extend to Canada balsam also. Slides cleared with trichlorethylene and mounted in balsam dissolved in this solvent dry very quickly, due to its higher volatility, thus making slides almost immediately available for use after preparation.

The solvent has been found especially useful for the salvaging of sections from cracked and dried slides. Cover glasses which are not removed by two weeks soaking in xylene are easily removed by an hour's immersion in trichlorethylene, without damaging the sections themselves. Due to the high specific gravity of the solvent (1.46) the sections float to the surface when the cover glasses fall off, and can be easily skimmed off and remounted. For slides in which the cover is not cracked but in which the balsam has dried away from the edge leaving a brown ring, a small amount of the solvent is run under the cover by means of a camel's hair brush, thus dissolving away the brown residue. A dilute solution of balsam in trichlorethylene is then run under the cover; any bubbles forming float to the edge and any excess balsam is removed after partial drying by brushing with pure solvent.

Another advantage of this solvent is the lower viscosity of its solutions with Canada balsam in the range of concentrations usually employed. Due to its greater density, the initial viscosity of trichlorethylene is higher than that of xylene, but the viscosity of solutions of Canada balsam in it are lower, i. e., its viscosity line is steeper than

[1]Buchner, Georg. Behavior of beeswax with trichlorethylene at ordinary temperatures. Chem. Ztg., 52, 319. 1928.

that of a balsam-xylene solution, and crosses the viscosity line of balsam-xylene below the range of concentrations usually employed. Thus, in trichlorethylene-balsam solutions an increase in concentration of balsam makes much smaller differences in the viscosity of the medium.

Objection to this solvent may be raised because of its slight narcotic effect. The author finds, however, that this effect is entirely absent unless the nostrils are applied directly with forced inhalation to the mouth of a bottle containing trichlorethylene, or to a cloth moistened with it. Furthermore, the only cases of poisoning reported as due to this solvent[2] have been caused by entry of industrial workers directly into large tanks which have contained trichlorethylene, and are hence saturated with its vapor.

The author acknowledges the assistance of Miss Esther Wilson in determining the extent to which this solvent may be employed in histological technic.

[2]Bridge, T. C. Industrial disease among chemical workers. (Ann. Rept. Sr. Med. Inspector). Chem. Age (London), **23**, 138–9. 1930.

THE FIXING AND STAINING OF LIRIODENDRON TULIPIFERA ROOT TIPS AND THEIR MYCORRHIZAL FUNGUS[1]

ISADORE COHEN, *Harrison Fellow, Department of Botany, University of Pennsylvania,* and K. D. DOAK, *U. S. Department of Agriculture, Bureau of Plant Industry, Office of Forest Pathology*

ABSTRACT.—**Root tips of *Liriodendron Tulipifera* forming with a fungus of the *Mycelium Radicis* group an endotrophic mycorrhiza, were subjected to several different fixations in which the action of cationic chromium and anionic chromium were compared. Anionic chromium in the form of chromic acid was combined with several substituted benzene compounds while cationic chromium in the form of chromic sulfate ($Cr_2(SO_4)_3 \cdot 15\ H_2O$) in 4% formaldehyde (HCHO) was used with the same ring compounds. In addition, several fixatives not containing chromium were tested.**

Five percent chromic sulfate ($Cr_2(SO_4)_3 \cdot 15\ H_2O$) in 4% formaldehyde (HCHO) or in 1% osmic acid with saturated aqueous salicylic and /or picric acid preserved the histological and cytological details of the mycorrhiza, as was clearly demonstrated when followed by staining in 3% acetic acid saturated with orseillin BB and counterstained with 1% crystal violet in clove oil.

Two percent ferric chloride ($Fe\ Cl_3$) in 4% formaldehyde showed the relationship of the tannin contents of the cells to the invading hyphae when followed by suitable staining.

Cationic chromium appeared to be superior to anionic chromium in preserving cell walls as well as the general histologic features of the material investigated.

INTRODUCTION

It is often desirable to fix plant tissue infected by fungi in such a way as to preserve not only the cell walls of both the host tissue and the invading hyphae, but also their cytological details. A useful general fixative should preserve the details of such an association so that they can be seen clearly when suitably stained. This fixation of pathological material has never been adequately treated in the handbooks of microscopic technic, for the probable reason that little experimental work has been done with such material.

[1]The senior author wishes to express his appreciation to the Allegheny Forest Experiment Station, maintained at Philadelphia, Pennsylvania, in coöperation with the University of Pennsylvania, for the support which made this investigation possible.

The purpose of this investigation was to obtain a suitable fixative for root tips of *Liriodendron Tulipifera* infected with a fungus of the *Mycelium Radicis* group (Doak, 1934c). The work was undertaken after it was learned that an acetic acid-formaldehyde-alcohol fixative and preservative (Doak, 1927) is not suitable for such material, especially when it is desirable to investigate the fine cytological details of the fungus and host elements, which is necessary in studies of mycorrhizal structures.

Material and Methods

Root tips of *Liriodendron Tulipifera* were collected March 16, 1934, from the study plots of the Pennsylvania Department of Forests and Waters located in the Westtown Friends' School Forest, Milltown, Pennsylvania. Random samples of the root tips from a large collection were fixed in the following fluids:

I. Acetic acid, formaldehyde, alcohol (Conant's Modified)

Glacial acetic acid	1.5	cc.
Formaldehyde (40%)	8.5	cc.
50% ethyl alcohol	90.0	cc.

II. Copper propionate, formaldehyde[1]

Propionic acid (C_2H_5COOH)	3	cc.
Formaldehyde (40%)	10	cc.
Water	87	cc.
Copper hydroxide ($Cu(OH)_2$)	excess	

III. Modified Erliki's[2]

Potassium dichromate ($K_2Cr_2O_7$)	1.25	g.
Ammonium dichromate ($(NH_4)_2\ Cr_2O_7$)	1.25	g.
Copper sulfate	1.0	g.
Water	100	cc.

IV.[3]

Chromic sulfate ($Cr_2(SO_4)_3 \cdot 15H_2O$)	5	g.
Formaldehyde (40%)	10	cc.
Water	90	cc.
Copper hydroxide	excess	

V. (a) 2% (by weight) ferric chloride in 4% formaldehyde
(b) 4% formaldehyde acidified with hydrochloric acid

VI. 2.4% sulphosalicylic acid ($C_6H_3OH\ (SO_3H)\ COOH$) with the following:
(a) 1% chromic acid
(b) 4% formaldehyde
(c) 5% chromic sulfate in 4% formaldehyde

[1]Zirkle 1931. [2]Zirkle 1934. [3]Zirkle 1928.

VII. Salicylic acid (C_6H_4OH COOH) sat. aqu. solution with the following:
  (a) 1% chromic acid
  (b) 4% formaldehyde
  (c) 5% chromic sulfate in 4% formaldehyde

VIII. 1% phenol (C_6H_5 OH) in the following:
  (a) 1% chromic acid
  (b) 4% formaldehyde
  (c) 5% chromic sulfate in 4% formaldehyde

IX. 1% resorcinol (C_6H_4 $(OH)_2$) in the following:
  (a) 1% chromic acid
  (b) 4% formaldehyde
  (c) 5% chromic sulfate in 4% formaldehyde

X. 1.2% pyrogallol ($C_3H_3(OH)_3$) in the following:
  (a) 1% chromic acid
  (b) 4% formaldehyde
  (c) 5% chromic sulfate in 4% formaldehyde

XI. Picric acid (C_6H_2OH $(NO_2)_3$) sat. aqu. solution in the following:
  (a) 1% chromic acid in 4% formaldehyde
  (b) 5% chromic sulfate in 4% formaldehyde

XII.
  (a) 5% chromic sulfate in 1% osmic acid (OsO_4)
  (b) Same as (a) with picric acid to saturation
  (c) Same as (a) with salicylic acid to saturation
  (d) Same as (a) 10 cc. with 2 drops glacial acetic acid
  (e) Same as (a) 10 cc. with 0.05% phenol

The time for fixation in each case was 48 hours. The root tips were dehydrated in the butyl alcohol series, sectioned 12 μ in thickness and stained chiefly with orseillin BB crystal violet (Doak, 1934a). The procedure was as follows:

3% acetic acid saturated with orseillin BB 30 minutes
Rinse in water
25–50–75–95% alcohols.....rinse
1% crystal violet in clove oil 5 minutes
Xylene..... wash
Xylene..... clear

The crystal violet solution was made by adding enough alcohol to 1 g. of dye to form a paste. Then the flask in which the solution was being made was gently heated to drive off the excess alcohol, and 100 cc. of clove oil were added. The crystal violet used was a product of Coleman and Bell Company, uncertified, but specified as C. I. 681 and as having a total dye content of 84%. The orseillin BB used was a product of Grübler, Leipzig, obtained in 1932.

DESCRIPTION OF PLATE I

These photomicrographs are taken from longitudinal sections of root tips of *Liriodendron Tulipifera* infected with a fungus of the *Mycelium Radicis* group and stained with orseillin BB and crystal violet. Fig. 3 is stained in safranin with no counterstain. Figs. 1 and 3 were taken with a Bausch and Lomb high dry, n. a. 0.65; Fig. 2 with a Spencer high dry n. a. 0.66; Figs. 4, 5, and 6 with a Zeiss oil immersion fluorite, n. a. 1.3. A Bausch and Lomb X6 ocular was used on all photomicrographs.

Figure 1. Fixed in Conant's Modified showing shrunken host cells and hyphae which are fixed as hollow structures. Magnification: X300.

Results

Fixatives containing a strong acid which seemingly penetrates ahead of formaldehyde preserves the hyphae as hollow structures, but with well-fixed nuclei. The fixing action of such combinations has been reported by Zirkle in several of his papers dealing with fixation. If, in addition to such a strong acid, relatively concentrated alcohol (50%)is incorporated in the fluid, the hyphae and the host tissue appear as described above, but the walls of the hyphae as well as the cell walls of the host are collapsed and shrunken (Fig. 1). Formaldehyde alone is useful, but the addition of other reagents improves its fixation image. Substances which increase the H-ion concentration of the formaldehyde, but which apparently do not penetrate the specimen ahead of the aldehyde generally give more useful images than those substances which decrease the H-ion concentration of the fluid. This observation is supported by a comparison between specimens preserved in a formaldehyde fixative containing the acid salt, ferric chloride, and those specimens preserved in a fixative containing the relatively basic substances, phenol, resorcinol, or pyrogallol (Fig. 3). A mixture of ferric chloride and formaldehyde has been used previously by other workers as a test for tannin.

Salicylic acid in all fluids tried gave useful images, but when combined with chromic acid (Cohen, 1934) it was not as satisfactory as when combined either with formaldehyde alone or with formaldehyde mixed with chromic sulfate in preserving the cell walls and the hyphae of the fungus (Figs. 2 and 6).

Figure 2. Fixed in salicylic acid and formaldehyde showing the fixation of the cell walls of the host tissue and the intimate association of the intracellular hyphae. The two cells on the right show the hyphae partly digested. Magnification: X354.

Figure 3. Fixed in ferric chloride and formaldehyde. The heavily colored, spherical bodies are tannins. The smaller spherical bodies within the hyphae are nuclei. Magnification: X294.

Figure 4. Fixed in chromic sulfate, formaldehyde, picric acid showing the cytoplasmic contents of the hyphae. Magnification: X660.

Figure 5. Fixed in chromic sulfate, formaldehyde, copper hydroxide showing the association of the cell walls of the host tissue and hyphal walls of the fungus. Magnification: X660.

Figure 6. Fixed in chromic sulfate, formaldehyde, salicylic acid showing the fine preservation of the hyphal walls of the fungus and the cell walls of the host tissue. Magnification: X660.

Most of the chromic sulfate-formaldehyde combinations preserved the root tips well enough for histological purposes, but those combinations to which the "basic" ring compounds were added did not preserve the cytological details of the host and fungus nearly as well as the combinations to which the "acid" ring compounds (salicylic and picric acids) were added. (Figs. 4 and 6). Salicylic and picric acids with 5% chromic sulfate in 4% formaldehyde gave an image close to the one desired. The cell walls of the host and hyphae were neither shrunken nor collapsed, and the finer cytological details in both elements of the mycorrhiza were well preserved. The two images illustrated in Figs. 4 and 6 are both primary images of formaldehyde modified by the "acid" ring compounds. The fixation image illustrated in Fig. 4 approaches that type of image in which mitochondria would be preserved, while in Fig. 6 the image illustrated represents one in which the mitochondria would be destroyed even if they were preserved in the primary fixation image of formaldehyde.

All the preparations fixed in fluids containing cationic chromium appear to be preserved better than those in which anionic chromium was used. The sole exception to this was the salicylic-chromic acid combination. Salicylic acid is notable in that even when used alone it preserved the cell walls better than any other single reagent tested.

No essential differences in the fixation images of material fixed in chromic sulfate-formaldehyde and in chromic sulfate-osmic acid mixtures were detectable. The chromic sulfate, osmic, acetic acid mixture (XIId) preserved the root tips of growing specimens collected at a later date, so that the mitotic figures were well fixed.

Altho several different staining combinations such as safranin and orseillin BB were studied, each with light and fast green, methyl blue, anilin blue (all in 95% alcohol) the finer details of the mycorrhiza were shown more clearly by staining in 3% acetic acid saturated with orseillin BB and counterstained with 1% crystal violet in clove oil. The cell walls of the host and the walls of the fungus were sharply stained by either one of the dyes. Where crystal violet replaced the orseillin BB a differentially stained preparation was obtained. The hyphae were colored red by the orseillin BB while the cortical cells were colored blue by the crystal violet. A difference in the staining reaction between the cells in the cortex and the central cylinder was observed. The crystal violet replaces the orseillin BB in the cell walls and nuclei of the cortex more readily than it does in the central cylinder. Thus, preparations were secured in which the walls and nuclei of the cortical cells were colored blue and their intracellular hyphae colored red, while cells in the central cylinder were stained by the orseillin BB

only. In some instances the hyphae within the cortical cells were stained by crystal violet. It is of no consequence whether orseillin BB or crystal violet stains the walls of the host and fungus as long as each is clear where the penetration occurs.

The safranin preparations were stained in a 1% aqueous solution, differentiated with a weak alcoholic picric acid mixture to which a few drops of KI-I were added, and finally counterstained with the various dyes in 95% alcohol, mentioned previously.

Discussion

An evaluation of the net differences in the fixing action between cationic and anionic chromium is difficult when so little is known of the chemical principles involved in the process of fixation. While fixation of cell walls essentially involves their preservation in as nearly the original condition as existed in the living tissue, the final evaluation of the specific action of the reagents in a fluid should not overlook the effect of treatment subsequent to fixation. Altho anionic chromium may fix the cell walls as well as cationic chromium, the former may not harden them sufficiently to withstand dehydration in the alcohols and infiltration with paraffin. The lack of resistance to dehydration of the structural elements of the walls (possibly the supermicelles observed by Seifriz with the Spierer lens (Seifriz, 1931)) may account for the observed superiority of material fixed with cationic chromium which apparently does harden tissue, over material fixed with anionic chromium. The action of alcohol in high concentrations is apparently one of dehydration which causes the collapse and shrinkage of the walls of the host and hyphae of the fungus.

Altho the chemical action of chromic sulfate in leather manufacture may be a direct union of the cation with the proteinaceous collagen fibers in the hides, similar union of chromic sulfate with the structural elements of the cellulose walls in plant tissue, perhaps conceivable altho to be sure not demonstrated by the work reported here, cannot be incorporated into any explanation of its specific action in a fixative.

There is no ready explanation of the action of salicylic acid on cell walls. More work with this acid is necessary before even an attempt at explanation is made for the effect of ring compounds in general in fixation has not been adequately investigated.

In addition to *Liriodendron Tulipifera*, in which the mycorrhizal fungus is endotrophic, several collections of pine roots in which the mycorrhizal fungus is ectotrophic were fixed. Unfortunately, the material was infected by secondary fungi and was in a state of degener-

ation preventing conclusive observations. Fixation studies of the ectotrophic mycorrhizal forms will be reported later.

Conclusions

1. The satisfactory preservation of plant tissue infected by fungi requires, in addition to proper cytological fixation, the undistorted preservation of both the cell walls of the host and hyphae of the fungi.

2. Five percent chromic sulfate ($Cr_2(SO_4)_3 \cdot 15\ H_2O$) in 4% formaldehyde saturated with picric or salicylic acids fixes such material so that details of the intimate association can be clearly observed. Salicylic acid appears to be especially useful for such work.

3. Cationic chromium appears to be superior to anionic chromium in cell wall fixation.

4. Three percent acetic acid saturated with orseillin BB and 1% crystal violet in clove oil is a useful histological stain combination for fungus-infected plant tissue.

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A SIMPLE METHOD FOR BLEACHING LEAVES

ILDA MCVEIGH, *Department of Botany, University of Missouri, Columbia, Missouri*

Many methods for bleaching leaves to show the general vascular anatomy are not entirely satisfactory. The method presented here is very simple and has been found useful in preparing leaves for starch tests for the course in general botany. It involves the use of a 5% sodium hypochlorite solution. Since sodium hypochlorite soon deteriorates, it is more satisfactory to use common household bleaching agents which contain approximately 5% sodium hypochlorite as the active principle. Such bleaching solutions can be bought at any grocery store and are ready for use. Some which have been used satisfactorily are Chlorox, Purex, and Sani-Clor.

Leaves to be tested for starch are first killed by boiling in water and then placed in the sodium hypochlorite solution and left until they are completely bleached. They are then washed in running water for 2 to 4 hours to remove this solution. Care should be taken to remove all of the bleaching agent, otherwise the iodine has no effect because it is also bleached. The color contrast in "ganonged" leaves is very distinct after this treatment. Leaves should not be left in the sodium hypochlorite solution longer than necessary as they become soft and easily torn, especially after having been boiled.

Fresh leaves may also be bleached by placing them directly in the bleaching solution. They can be "wetted" more easily by first rinsing them in 95% alcohol. The length of time required for bleaching varies with the thickness and texture of the leaf. Thin leaves and leaves of some water plants often require no more than 30 to 60 minutes, whereas thick leaves, such as those of Bryophyllum, require from 24 to 96 hours. The bleaching of thick leaves is hastened by splitting the lower surface along the midrib or by removing a portion of the epidermis in this region. Such treatment allows the bleaching solution to penetrate more readily and yet does not obscure the vascular structure of the leaf.

In order to render the vascular anatomy still more visible the leaves are dehydrated in alcohol and then stored in a glycerin-alcohol solution (75% glycerin, 25% alcohol). As soon as the leaves have become impregnated with glycerin they may be removed from the solution and the excess glycerin removed with filter paper. The leaves are pliable and thus easier to handle and study in this condition, and they keep this way for several weeks.

Photographs to show the vascular anatomy can then be made by using the leaf as a negative and projecting the image directly onto sensitized paper which is then developed in the usual way. The photographs can be made by means of an ordinary photographic enlarging apparatus. It is better to photograph the leaves soon after they are removed from the glycerin-alcohol solution. If pen and ink drawings are desired they can be made from the photograph according to the method of Naylor[1] to whom acknowledgment is made for suggesting the technic.

This method for bleaching leaves is just as useful for dried (herbarium) material and material preserved in formalin-alcohol solution as for fresh material. It is not satisfactory for material which is to be used in cytological studies since the cell contents are plasmolyzed and somewhat destroyed.

[1]Naylor, E. E. Pen and ink drawings from photographs. Science, **73**, 15.

NOTES ON TECHNIC

METHYLENE BLUE AND ACID FUCHSIN FOR SUBCUTANEOUS TISSUE SPREADS.—Difficulty is often encountered in the preparation of consistently thin connective tissue spreads and uniform good differentiation of cellular elements for class room use. Shrinking of the elastic fibers by prolonged fixation and dehydration frequently thickens the spread to such an extent as to interfere with staining procedures. Uneven staining, stained backgrounds, and poor differentiation are the results. The following technic, which in many respects is a modification of the classical method, gives excellent results.

1. Inject a white rat subcutaneously in the pectoral region with 1 cc. of 1% trypan blue in distilled water each day for four days, or until the mucous membranes and skin are a distinct blue.
2. Anaesthetize the animal and remove small bits of subcutaneous tissue from the groin region. Avoid removing any bits of fat or muscle with the tissue.
3. With the help of an assistant stretch the tissue as thin as possible on a clean slide with dissecting needles. Hold the tissue in place until the edges become dry and adhere to the glass.
4. Fix in 5.0% neutral formalin 30 minutes to 1 hour.
5. Wash thoroly in distilled water.
6. Stain 1 minute in 1% aqueous methylene blue (a Grübler sample used).
7. Rinse thoroly in distilled water.
8. Counterstain 30 seconds in 1% aqueous acid fuchsin (a Coleman and Bell uncertified sample used).
9. Rinse thoroly in distilled water.
10. Dehydrate rapidly in acetone.
11. Differentiate in absolute alcohol.
12. Clear in xylene, mount in Canada balsam.

Mast cells show heavy blue granulations often obscuring the light blue nuclei; histocytes have fine blue granulations in cytoplasm, nuclei blue; eosinophiles, red granulations, blue nuclei; fibroblasts, pale blue homogeneous cytoplasm and pale blue nuclei. Connective tissue fibers stain in various shades of red according to type.

Preparations, if not exposed to heat or strong sunlight, will not fade for three to four years.—THELMA T. BAIRD, Ohio State University, Columbus, Ohio.

LUGOL'S SOLUTION FOR THE FLEMMING TRIPLE STAIN. The application of alcoholic Lugol's solution after safranin and after the violet

seems to improve and to stablize as well as to hasten the differentiation of the Flemming triple stain for botanical material such as root tips and buds.

The following procedure was used for material fixed in Flemming's or Ruffini's solutions, (the Ruffini fixed material was embedded thru terpineol): staining 1½ to 3 hours in 0.5 to 1% safranin, rinsing in distilled water, treating with Lugol (1% iodine and 1% potassium iodide in 80% alcohol) for 15 to 25 seconds, rinsing in distilled water, staining for 5 minutes in 1% violet, rinsing in distilled water, treating with Lugol for about 30 seconds, dehydrating with absolute alcohol and differentiating with clove oil and orange G. In this work the following stains were used: Safranin, Cert. No. NS-8, and a mediocre, uncertified batch; crystal violet, Cert. No. NC-15, a Grübler gentian violet, and a methyl violet 2B, Cert. No. NMv-4; orange G, Cert. No. CO-4.

Flemming's triple stain may readily be used after Navashin's fixation and the procedures usually following it. In this case, proceed as above except for leaving out the iodine treatment after the safranin.—Lubow A. Margolena, Geneva, N. Y.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked.

EVANS, N. and KRAJIAN, A. New method of decalcification. Arch. Path., **10**, 447. 1930.

Method can be used on material fixed in 1:10 formalin or in other standard fixatives. Decalcifying solution consists of 1 part 95% alcohol, 1 part 85% aqueous formic acid solution, 1 part 20% aqueous sodium citrate. Soak from a few hours to 3 to 4 days. After decalcification is completed, wash tissue in running water for 16 to 24 hours. Cut by freezing or paraffin method. Staining properties, especially of nuclear elements, are said to be unaffected.

FIELDING, J. W. The staining of acid-fast bacilli. The effects of fixatives and H-ion concentration on acid-fastness. Austral. J. Exp. Biol., **12**, 1–5. 1934. (Abstr. in J. Royal Micro. Soc., **54**, 121. 1934.)

Fixation of tissue in formalin for as long as 20 years does not affect the acid-fastness when alkaline fuchsin was used as a stain. Neither acetone nor a combination of acetone and formalin is responsible for loss of acid-fastness. Autolytic action in tissues is responsible for lowering pH value of the fixative. Low pH values are responsible for the change in the staining reaction of acid-fast organisms. If the pH of the fixative is kept on the alkaline side or staining is carried out with an alkaline fuchsin there is no loss of acid-fastness.

HOLLISTER, GLORIA. Clearing and dyeing fish for bone study. Zoologica, **12**, No. 10, 89–101. 1934.

(1) Fix specimen, if necessary, as with large fish, in 70% alcohol. (2) Rinse in distilled water few minutes. (3) Soak in 1 to 4% KOH, varying concentration and time in relation to size and consistency of specimen. (4) Stain, for such length of time as seems necessary for the individual specimen, in an alizarin solution prepared as follows: 1 cc. of a saturated solution alizarin red S (in 5 parts 50% acetic acid, 10 parts glycerin, and 60 parts 1% choloral hydrate) mixed with 1000 cc. KOH, which varies in strength from 1 to 4%, according to the nature of the specimen. Various brands of alizarin red S have been tried with equal success. (5) Clear in 1 to 20% KOH, with or without glycerin, according to individual. Irradiate with ultra-violet light. (6) Continue irradiation with specimen in KOH, adding glycerin by degrees. (7) Preserve specimens (soft tissues clear, bone tissue stained red) in glycerin with a thymol crystal in a glass or rubber topped jar.

HOOK, H. T. and BRIGGS, BERNICE. Iodine-potassium-iodide, as a fixative and differential stain. Science, **80**, 142–3. 1934.

Iodine-potassium-iodide, (prepared according to the usual formula of 1 g. of iodine crystals, 2 g. of potassium iodide and 300 cc. of water) was found to be useful as a fixative and a differential stain in studies on the germination of conidia of the Peronosporales.

Use a drop of the iodine solution for every drop of the spore suspension. This almost instantly kills the conidia and stains the ungerminated ones a golden brown. Germ tubes of the recently germinated conidia are stained a golden brown, but empty spore cases remain colorless. In the more advanced stages of germination the germ tubes do not stain at all, or only at the tip.

The differential properties of the stain are apparently due to the fact that only the protoplasm is stained, the conidial and germ tube walls remaining uncolored. The method has been successfully used on *Peronospora parasitica*, *Peronospora lami* and *Plasmopara gerani* and on dried specimen of *Pernonspora destructor*, *Bremia lactucae*, *Basidiophora Kellermani* and *Albugo candida*.

JOHN, C. Über meine Ergebnisse bei der Darstellung von Nerven-elementen durch das Ankohlungsverfahren. Zts. Zellforschung, Mikr. Anat., **18,** 593–5. 1933. (Abstr. in Zts. Wis. Mikr., **50,** 368–9. 1934.)

The author finds that by a method similar to Kisser's "Ankohlungsverfahren" nerve elements may be satisfactorily revealed.

Mount frozen sections on a large drop of water. Heat the sections slowly over a Bunsen burner until they become dark yellow-brown, taking care to prevent charring. Control under the microscope. Cool and mount in balsam. Paraffin material is treated similarly, except that the paraffin is first dissolved and the sections are run down thru alcohols to water prior to heating.

The preparations resemble Bielschowsky's impregnated material and are very well suited for diagnostic purposes. Good results were thus obtained with genitalia, liver, kidney, and especially with the central nervous system. Lymph glands, spleen, tonsils, fatty tissue, bone marrow, and smooth muscle are unsuitable for this procedure.

MANEVAL, W. E. Rapid staining methods. Science, **80,** 292–4. 1934.

(1) Negative Staining of Microorganisms: One percent nigrosin or 2% Congo red is frequently used in the negative staining of microorganisms, sometimes changing the color of Congo red to blue by adding a drop of 1% HCl in 95% alcohol to the dried suspension on the slide. A 1% solution of rose bengal, Magdala red, or cotton blue may be added to a 1% nigrosin in proportion of 1 to 3, or 30 drops of 1% acid fuchsin and 4 drops of concentrated HCl to 40 cc. of 1% nigrosin. Traces of acids may be added to the nigrosin-cotton-blue; a little alkali may prove advantageous with rose bengal or Magdala red. After drying, the films of any of these combinations may be fixed with 2% ferric chloride for 1 minute, then washed with water. (The writer does not give the source of the dyes nor state whether he employs *true* Magdala red or the less expensive variety of "Magdala red" which is really phloxine.)

The nigrosin-cotton-blue combination is good as a background for stained mounts of vegetative forms and spores of bacteria and fungi, protozoa, etc. Rose bengal or Magdala red with nigrosin are recommended for bacteria and fungi. Cells suspended in the dye mixture appear practically colorless, but if stain is added to a *dry* smear and allowed to dry, vegetative cells will be pink or red. Bacteria with Magdala red show pink cytoplasm and colorless spores. Nigrosin-acid-fuchsin acts the same way on bacteria, staining early yeast spores pink.

(2) A Rapid Staining Method for Dividing Cells: Used on root tips and anthers of *Lilium* (chromo-acetic or Flemming's fixation). Run paraffin sections thru xylol, absolute, 95, 70, 50 and 30% alcohol and water. Pipette 2 or 3 drops of each on slide, let it act 20 to 30 seconds and drain. Stain 3 to 5 minutes with anilin-alcohol-fuchsin. (See Stain Techn. **4,** 21–25, 1929.) Wash 3 or 4 times in water, dehydrate with 95% alcohol containing a trace of HCl (for destaining), 95% and absolute alcohol. Counterstain with 0.1% solutions of orange G and light green in clove oil (1:3). Wash with absolute alcohol, clear in xylol, mount in balsam. Anilin blue in 90% alcohol is equally recommended as counterstain. Procedure requires about 8 minutes; is not wasteful. The light green tempers the orange color, imparts a greenish shade lasting for months. Crystal violet may be substituted for basic fuchsin in above formula, or both dyes may be used, first crystal violet, followed by basic fuchsin after rinsing in water.

Safranin can be used instead of basic fuchsin in a similar formula: Safranin, 3%, in absolute alcohol, 20 cc.; distilled water, 20 cc.; alcohol, 95% (3 parts) and anilin oil (1 part), 5 cc.; (acetic acid, 4%, 1 cc., may be added). Stain 3 to 10 minutes, wash with water, counterstain with 0.5% cotton blue in 70% alcohol for a few seconds, dehydrate, clear and mount. (The writer does not state which of the various cotton blues is employed; the usual dye thus named is the same as anilin blue W. S.) The anilin-alcohol-safranin and the anilin-alcohol-basic-fuchsin (3 minutes) followed by Delafield's hematoxylin were found satisfactory for stem and leaf sections.

MARTENS, P. Etude expérimentale des sporocytes de Tradescantia—II. Action du "suc" de la plante sur les cellules vivantes. Zts. Wis. Mikr., **51,** 88–102. 1934.

In attempts to find a suitable medium for studying living cells other cytologists have tested cell sap (of the organism in question), hoping that this "natural" medium would prove satisfactory. Effects of the influence of the sap on the various nuclear stages are here described. A review of results obtained by other investigators is given.

From observations made on sporogonial cells, sporocytes, and tetraspores of *Tradescantia virginica*, the author concludes that the sap causes nuclear contraction, loss of some caryolymph material, and an irreversible coagulation of chromosomes and nuclear structures. Ringer's solution also influences cell appearance, but to a much lesser degree, and the modification is reversible, for when transferred to an isotonic sugar solution the chromosomal structures, etc. gradually disappear.

McArthur, J. N. A new type of portable microscope. J. Royal Micro. Soc., **54**, 182–5. 1934.

The instrument represents a somewhat radical departure from the conventional design of the standard microscope. Altho it serves the functions of the latter, it has greater rigidity and simplicity along with lesser weight and size, in spite of being designed in pocket size.

Essentially, this compact instrument consists of an eyepiece at one end to which the image arrives by way of two reflecting prisms underneath. One to three objectives, looking upward, are mounted on a sliding plate and can be easily slipped between object and the first reflecting prism at the other end. The object lies above the objective on the under side of the slide. Above it a hinged mounting carries condenser and iris diaphragm. A hinged horse-shoe base can be slipped out from alongside the light tube on the bottom.

Claimed advantages: (1) Coarse adjustment eliminated, fine adjustment minimized. (2) No breaking of coverslips. (3) Full direct vision of object at any time. (4) Lying-drop preparations possible. (5) Live-box and all manipulations permitted with oil immersion. (6) Accessible condenser; vertical and unreflected lighting. (7) Objectives easily interchanged and accurately centered. (8) Greater rigidity and less wear and tear on the focusing mechanism.

Mishulow, Lucy, Melman, Mildred and Romano, Marie. An improved method of direct smear examination for acid-fast bacilli in sputums. Science, **80**, 143–4. 1934.

The usual method of examining sputa for acid-fast organisms by taking a loopful from the sputum jar frequently gives negative results when the number of organisms is comparatively small. The authors found that instead of resorting to concentration of the sputum, which is the usual procedure, careful selection of the material and thoro examination are time-saving and entirely reliable measures.

Proceed as follows: Pour the sputum into a Petri dish, and examine for mucus flakes. If the sputum is fluid, collect the flakes with a pipet and expell on two chemically clean slides, making fairly thick smears. If the sputum is very mucoid, use a heavy wire loop instead of the pipet. Dry in the air and stain with Ziehl-Neelson fuchsin, decolorize, etc. Counterstain with 1–1000 dilution of brilliant green, instead of methylene blue.

Neild, H. W. A rapid method for the preparation of Delafield's haematoxylin. Science, **79**, 209–10. 1934.

The usual ripening of hematoxylin may be hastened to 3 hours by means of the following method. Dissolve 4 g. hematoxylin crystals in 25 cc. 95% alcohol and 400 cc. saturated solution of ammonia alum. Place this solution in an open dish, at a distance of 15 cm. from a Cooper-Hewitt burner, operating at 140 volts and 3.3 amperes, for 1 hour. Filter the solution and add 100 cc. of methyl alcohol, and 100 cc. of glycerin. Place the solution under the same burner for 2 hours. After filtering, the stain is ready for use. No appreciable difference was noticed between the staining quality of the hematoxylin prepared in this manner and that left for 60 days to ripen.

Rettie, T. A new method of applying Nile blue as a fat stain. J. Path. & Bact., **34**, 595–6. 1931.

When Nile blue sulfate is boiled with dilute sulfuric acid it is partially converted into oxazone diethylaminophenonaphth-oxazone, which is soluble in fats and fat solvents, giving a pink solution. It is this pink oxazone that is the fat stain, and the author calls it "Nile pink".

Prepare Nile pink by dissolving 3 g. Nile blue A in 500 cc. water, adding 3 cc. sulfuric acid and 100 cc. xylol. Heat the mixture on a water bath with continuous shaking. The Nile pink dissolves in the xylol and is recovered by distilling off the xylol. Use a saturated solution of Nile pink in alcohol for the stock solution. Mix 2 cc. of the stock with 1 cc. of a 20% sulfuric acid, 7 cc. 5% of dextrin, and add 0.2 cc. of a 0.5% Nile blue. If water is used for dilution instead of the dextrin the stain crystalizes out within an hour, while with dextrin it keeps for several days.

Frozen sections stained ½ to 1 hour, washed in water, and mounted in glycerol or farrant solution show all fat bright pink, against a grey-blue background. In staining very fine fat granules as in degenerated tissue, Nile blue should not be added to the solution; by raising the condenser the finest pink granules can be easily observed. For demonstrating fat in fresh or fixed thin layer tissue (diaphragm, omentum), use 1 cc. of the stock, 2 cc. 20% sulfuric acid, and dilute with water to 40 cc.

STOCKWELL, P. A stain for difficult plant material. Science, **80**, 121–2. 1934.

The following modification of Flemming's triple stain has been worked out for material that was unsuitable for the usual procedure.

If the fixative used contains no chromic acid, soak for 1 hour or over night in 1%. Rinse several times in tap water, and stain 1 to 24 hours in the following: 1 part 1% aqueous gentian violet, 2 parts 1% aqueous safranin, and 1 to 4 parts distilled water. A dilute stain for 24 hours gives best results. Wash in tap water and transfer for 30 seconds to 1% iodine-potassium-iodide in 70% alcohol. Run up thru two jars of 50% alcohol, one each of 70% alcohol, 95% alcohol plus picric acid (about 1%), 95% alcohol plus ammonia (8 to 10 drops per 100 cc.), 95% alcohol, and 100% alcohol. Differentiate with 0.2% orange G in clove oil, followed by clear clove oil, and pass thru three jars of xylol.

If too much safranin remains in the sections, remove it by immersing the sections in 100% alcohol; if the violet is in excess, differentiate again in clove oil.

Chromosomes vary in color from light to dark purple, depending upon their stage of development. Spindles show a different shade of purple, the nucleoli red, and the cytoplasm orange-yellow. Anatomical structures are stained the same as with Flemming's triple stain.

ZOTTNER, G. Coloration simple, sûre et rapide des corpuscules de Negri dans les coupes. Compt. Rend. Soc. Biol., **115**, 593–4. 1934. (Abstr. in J. Royal Micro. Soc., **54**, 121. 1934.)

Paraffin sections of the horn of Ammon are passed thru xylol to absolute alcohol; without washing, a few drops of a solution of nitric acid (1 in 3) are placed on the section and left for 2 to 3 minutes. The acid is then drained off and a few drops of Ziehl's fuchsin are run over the section for 15 seconds. Wash rapidly and allow the following freshly prepared mixture to stain for 15 seconds: equal parts of a saturated aqueous solution of picric acid and 1% indigo carmin; dehydrate and differentiate in absolute alcohol. As soon as the sections begin to take a greenish tint, clear in xylol and mount in Canada balsam. The Negri bodies are red or ruby-rose in color, neuroglia bright green, cell protoplasm green or purplish green, nucleoli and chromatin deep brown or garnet color.